An Approach for Screening Cholinesterase Inhibitors In Drinking Water

Kim R. Rogers[1], Nirankar N. Mishra[1], S. Twomey[1], and Amal Mahfouz[2]

[1] U.S. EPA, Office of Research and Development, National Exposure Research Laboratory, Human Exposure & Atmospheric Sciences Division, Human Exposure Research Branch, P.O. Box 93478, Las Vegas, NV 89193-3478, USA

[2] U.S. EPA, Office of Water, Office of Science and Technology, Washington, DC, USA

1 Abstract

Under the 1996 amended Safe Drinking Water Act (SDWA), a Contaminant Candidates List (CCL) has been compiled in 1998, FR 63 (40). Several cholinesterase (ChE) inhibitors were identified in this list, some are organophosphates and others are carbamates. Previous to 1996, ChE inhibitors in drinking water were regulated individually and monitored by using conventional analytical methods to detect the presence and concentration of individual contaminants. Because of specific advances in analytical chemistry and the need for cumulative risk assessment of chemicals that have a common mechanism of toxicity, we developed an approach for initially screening for the presence of ChE inhibitors in drinking water. This approach is based on determining the extent that spiked or contaminated drinking water inhibits ChE activity.

For the reported ChE inhibitor screening assay, the enzyme was stabilized in a gelatin film. The remarkable properties of the dry immobilized ChE preparation include its stability to prolonged storage at room temperature as well as its stability to short term elevated temperatures (60°C). The enzyme could be maintained in dry gel form for 365 days at room temperature without substantial loss of activity. Several procedures were evaluated to oxidize less potent P=S organophosphate compounds to their more inhibitory oxon forms. Inhibition profiles were run for six commonly used carbamate insecticides (and some of their metabolites) and eight organophosphate insecticides using this assay. The concentrations that cause 50% inhibition of ChE (IC_{50} values) were determined for purified water and several drinking water matrices. Results using this assay were also compared to a commercially available test kit from EnviroLogix™.

2 Background

One of the means of reducing uncertainties in the assessment of human exposure is to better characterize concentrations of hazardous compounds that may be present in our immediate environment. A significant limitation to this approach, however, is that sampling and laboratory analysis of contaminated environmental and biological samples, can be slow and expensive; thus, limiting the number of samples that can be analyzed within time and budget constraints. Faster, simpler, and more cost-effective field screening methods can increase the amount of information available concerning the location, source and concentration of pollutants present in the environment. Among the compounds of interest to the EPA for human exposure assessment are pesticides. More specifically, insecticides from the organophosphates (OP) and carbamate classes are widely used in industrial and residential settings.

The toxic effects of OP and carbamate insecticides are mediated primarily through disruption of cholinergic neurotransmission by inhibition of acetylcholinesterase (AChE). In addition to their acute toxicity due to inhibition of AChE, some of these compounds have also been implicated with long term neurological problems such as delayed neurotoxic effect.

OP and carbamate insecticides vary considerably in their overall toxic behavior due to many factors, including absorption, metabolism and interaction of parent compounds and metabolites with various target and non-target proteins. Metabolic activation is particularly important for the phosphorothionates, a subclass of OP compounds which are characterized by sulfur (P=S) attached to the central phosphorous atom. For this class of insecticides, the parent compound typically shows little anticholinesterate activity whereas the oxidative metabolite (the oxon, P=O) is often highly potent.

Although there have been a variety of chromatographic methods reported for detection of OP and carbamate insecticides, these techniques are typically expensive and time-consuming. A wide variety of bioanalytical and biosensor methods based on inhibition of AChE have also been reported over the past decade. Some of these techniques have resulted in commercially available test kit assays. The focus of this report is to identify screening methods for mixtures of these compounds in water and to outline characteristics of a microwell plate assay method developed in our lab and compare these results with those using the EnviroLogix™ test kit assay.

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3 Summary and Conclusions

The first difference between the two assays is the form and concentration of the oxidant for conversion of the phosphorothioate OPs to more active forms. The EnviroLogix™ method uses 0.01% Br_2 in 10 mM NaBr, whereas the herein reported assay method uses 0.001% Br_2 or 0.01% NaOCl in phosphate buffer. The advantage to the higher Br_2 concentration for the EnviroLogix™ assay is that it would be expected to oxidize the phosphorothioates to a greater extent in the presence of matrix organics. The primary limitation is that in the absence of oxidizable organics, we have observed that the Br_2 is often not completely neutralized by the included reagent causing inhibition of the enzyme and leading to false positives. This may be a significant problem when the contaminating compounds are not known (e.g., mixed OPs and carbamates).

The next difference between these assays is that the herein reported method uses kinetic enzyme analysis whereas the EnviroLogix™ assay uses endpoint analysis. The main advantage to kinetic analysis is that it tends to be more sensitive to enzyme inhibition and consequently measures lower inhibitor concentrations. It should be noted here that the EnviroLogix™ kit met the reported detection limits for the compounds tested when run following the insert instructions. When the EnviroLogix™ assay was modified for kinetic analysis, however, the observed detection limits were typically at least an order of magnitude lower (particularly for the carbamates) than for the endpoint analysis. The main limitation for kinetics analysis is that to our knowledge there are no inexpensive field portable microwell plate readers that offer kinetics analysis capabilities.

We conclude that both the microwell plate assay and the EnviroLogix™ assays may provide an inexpensive approach to screen for the presence of ChE inhibitors in drinking water.

Recommendations:

The herein reported microwell plate assay yielded the following characteristics:

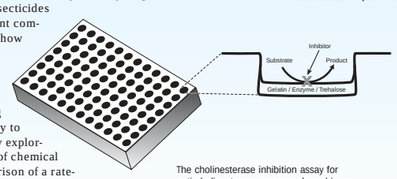
- Fast (approx 60 min)
- High Throughput (96 assays / plate)
- High Sensitivity (especially for the most potent of cholinesterase inhibitors)
- Inexpensive ($0.08 / assay)

The EnviroLogix™ assay yielded the following characteristics:

- Fast (approx 60 min)
- High Throughput (96 assays / plate)
- Sufficiently Sensitive (for a number of insecticides)
- Relatively Inexpensive ($2.50 / assay)
- Met all advertized claims

4 Introduction

Screening assays using surrogate cholinesterase are typically sensitive, reliable and have been extensively reported in the literature. Several versions of this assay are also commercially available. There are, however, certain limitations for the application of these assays to environmental monitoring. These limitations include variability of assay responses to various OP and carbamate insecticides (particularly the parent compounds that tend to show lower sensitivity than their oxidative metabolites). Work on this project has focused on increasing sensitivity of the assay to parent compounds by exploring various methods of chemical oxidation and comparison of a rate-based 96 well plate assay developed in our lab to a commercially available anti-cholinesterase screening assay.



The cholinesterase inhibition assay for anti-cholinesterase compounds and insecticides has been adapted to a 96 well micro-plate format.

5 Methods

Enzyme Immobilization and Insecticide Assay Protocol

Acetylcholinesterase (AChE) was dissolved in a solution containing 5% D-(+)-trehalose dihydrate, 5% D-(+)-glucose, 0.1% of gelatin, 1% sodium chloride and 0.002% of sodium azide (TGG) and distributed into individual wells of the plate. The enzyme was dried under a stream of air for 24 hrs at 25°C, after which it was ready for use in the inhibition assay. Immediately prior to the assay, the AChE was dissolved in phosphate buffered saline (PBS) containing 10 mM sodium phosphate, 100 mM NaCl, pH 7.4. AChE activity was measured using the Ellman method. In short, the reaction medium contained 75 µL of 1 mM acetylthiocholine chloride, 75 µL of 1 mM 5,5'-dithiobis (2-nitrobenzoic acid), 25 µL of AChE (2.86 ng) and 25 µL of DI water at pH 7.4. For the inhibition assay without prior oxidation of the inhibitors, 25 µL of AChE was incubated for 20 min with 25 µL of the inhibitor at concentrations ranging from 1 mM - 1 nM. For the assay variation that employed oxidation of pesticides containing P=S groups, the inhibitors (25 µL) were first incubated in the presence of 0.001% Br_2 or 0.01% NaOCl for 20 min followed by the addition of ethanol to a final concentration of 5% prior to the incubation with AChE.

6 Results

Enzyme Stability

One of the drawbacks for the use of enzyme-based assays for environmental monitoring has been the storage requirements (e.g., typically below 4°C) and limited shelf life. We report that the use of trehalose, glucose, gelatin, sodium chloride and sodium azide (TGG) for dried acetylcholinesterase (AChE) preparations dramatically stabilizes activity of this enzyme. The enzyme activity rapidly degraded in water or phosphate buffered saline (PBS) solution. Although the TGG allowed for maintenance of activity in solution over several days, AChE dried in this mixture remained active for extended periods of time (i.e., 1 year) at 25°C or for short periods at 60°C.

Stabilization of Acetylcholinesterase

Storage Media	Storage Temp	Storage Time	Activity (%)
DI Water	25°C	10 min	100
DI Water	25°C	3 hr	0
PBS	25°C	10 min	100
PBS	25°C	3 hr	0
TGG (soln)	25°C	3 days	75
TGG (dried)	25°C	15 days	100
TGG (dried)	25°C	60 days	100
TGG (dried)	25°C	365 days	100
TGG (dried)	60°C	10 min	100

Microwell Plate Assay

Characteristics for the Cholinesterase Inhibition Microwell Plate Assay

	IC_{50} (µg/L)*	MCLG or HA**
Carbamates		
Aldicarb	10	7
Aldicarb sulfone	16	7
Aldicarb sulfoxide	NR	7
Carbofuran	6	10
Oxamyl (vydate)	75	20
Baygon (propoxur)	202	3
Carbaryl	8	700
Methomyl	81	200
Organophosphates		
Diazinon	47	0.6
Disulfoton	652	0.3
Fonofos	NT	10
Terbufos	548	0.9
Chlorpyrifos	25	20
Fenamiphos	NT	2
Malathion	7	10
Parathion (methyl)	45	2

*Concentration of compound that results in 50% inhibition of the enzyme activity

**MCLG, Maximum Contaminant Level Goal; HA, Health Advisory

EnviroLogix™ Assay

Characteristics for the EnviroLogix™ Cholinesterase Inhibitor Microwell Plate Assay

	%Inhibition	*Conc(µg/L)	**IC_{xx}
Carbamates			
Aldicarb	0	1	NL
	23	**10**	
	33	**100**	
Aldicarb sulfone	0	1	NL
	0	10	
	8	100	
Aldicarb sulfoxide	19	**100**	NL
Carbofuran	2	1	
	10	10	
	25	**100**	100
	67	**1000**	
Oxamyl (vydate)	11	1	NL
	17	**10**	
	20	**100**	
Baygon (propoxur)	2	1	NL
	8	10	
	24	**100**	
Carbaryl	0	1	
	0	10	
	18	**100**	100
	78	**1000**	
Methomyl	0	1	NL
	0	10	
	0	100	
	%Inhibition	Conc(µg/L)	IC_{xx}
Organophosphates			
Diazinon	36	**0.50**	
	57	**5.0**	5.0
	100	**50**	
Disulfoton	NT	NT	NL
Fonofos	NT	NT	NL
Terbufos	28	**1**	NL
	54	**10**	
	100	**100**	
Chlorpyrifos	0	0.05	
	19	**0.5**	0.5
	80	**5.0**	
Fenamiphos	NT	NT	NL
Malathion	0	10	
	29	**100**	100
	100	**1000**	
Parathion (methyl)	0	50	
	98	**500**	500
	100	**5000**	
Control	0	0	
Kit Blank	69	NL	
Low Pesticide Std (kit)	46	NL	
High Pesticide Std (kit)	87	NL	

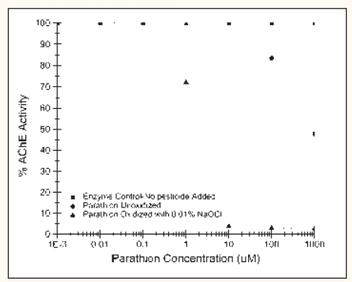
*Concentrations that inhibit the enzyme greater than 15% (i.e., detection limit) are listed in **bold**

**IC_{xx} listed in the kit insert (concentration of compound that results in 15% inhibition)

NL not listed
NT not tested

Pesticide Oxidation

The oxidative metabolites of many organophosphorus insecticides are significantly more potent inhibitors of AChE than their parent compounds. In order to make this assay more sensitive with respect to the potential use for screening environmental samples, the phosphorothioate OP insecticides (P=S) were converted to their oxon (P=O) derivatives. In the case of potential environmental application, there are several issues that must be considered. First, it is important to oxidize a reasonable percentage of the phosphorothioate. Next, because AChE is sensitive to halogenation, it is important to inactivate the oxidant prior to incubation of the sample with the enzyme. We found that a pre-treatment protocol involving exposure of the phosphorothioates to either bromine or hypochlorite followed after 20 min by 5% ethanol was relatively fast, efficient and did not result in inhibition of the enzyme. Analysis of the oxidation of parathion using hypochlorite by GC/MS showed a 24±3% conversion to the oxon derivative.



Notice: The U.S. Environmental Protection Agency (EPA), through its Office of Science and Technology (OST) in the Office of Water (OW) and the Office of Research and Development (ORD), initiated this investigation to screen for these CCL contaminants in drinking water. ORD has funded this research and approved this abstract as a basis for presentation. The actual presentation has not been peer reviewed by EPA. Mention of trade names or commercial products does not constitute endorsement or recommendation by EPA for use.